CHARACTERIZATION OF THE BIOLOGICALLY ESSENTIAL DOMAIN IN THE N-TERMINUS OF THE HUMAN TELOMERASE CATALYTIC SUBUNIT

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Breast cancer cells acquire the tumorigenic phenotype of unlimited proliferation through the activation of telomerase, the enzyme that elongates telomeres. Telomerase is composed of an RNA (hTR) subunit and a protein (hTERT) subunit. Normal somatic cells lose telomeric DNA, which ultimately limits their lifespan. In the vast majority of breast cancers the hTERT gene is upregulated, restoring telomerase activity, and thereby overcoming the proliferative block imposed by telomere shortening. Inhibition of telomerase activity in breast cancer cell lines results in cell death. Therefore, antagonizing hTERT function may serve as a specific means to treat breast cancer patients. Understanding how this enzyme functions and how it is regulated will be critical in developing methods to inhibit telomerase activity.

Telomerase activity can be detected throughout the cell cycle, whereas telomere elongation occurs only during S-phase. These data indicate that telomerase is regulated in vivo. To identify domains of hTERT required not only for catalysis, but also for in vivo regulation, we introduced a panel of 90 N-terminal hTERT substitution mutants into telomerase-negative cells and assayed the resulting cells for catalytic activity and, as a marker of in vivo function, cellular proliferation. We found four domains to be essential for in vitro and in vivo enzyme activity, two of which were required for hTR binding. Interestingly, we also discovered a novel domain that dissociates activities of telomerase (DAT), in which mutations left the enzyme catalytically active, but unable to function in vivo. Since mutations in the DAT domain neither decrease catalytic activity, nor prevent the enzyme from entering the nucleus, we suspect this domain may be involved in guiding telomerase to the telomeres. Current research will focus on testing this hypothesis.

The identification of the described domains of hTERT essential for enzyme function provides the first insight at how the enzyme is regulated in vivo, and ultimately will be of value in designing methods to inhibit the enzyme for clinical use in the treatment of breast cancer.

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PROCOLLAGEN C-TERMINAL PROTEINASE ENHANCER IN BREAST TUMOR CELL LINES

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Matrix Metalloproteinases (MMP) are a family of zinc-dependent proteinases that degrade components of the extracellular matrix (ECM). MMP are important for remodeling ECM during growth and development. Increased MMP activity has also been associated with tumor cell invasion, metastasis and tumor associated angiogenesis. MMP activity is inhibited by Tissue Inhibitors of Metalloproteinases (TIMP). However, increased TIMP has been associated with a poor outcome in some breast cancer patients. Recently, we identified the C-terminal fragment of Procollagen C-terminal Proteinase Enhancer (PCPE) as a non-TIMP inhibitor. CT-PCPE inhibitory activity has been observed in medium conditioned by the aggressive breast tumor cell line MDA MB-231 but it has not been observed in medium conditioned by normal cells or those from fibroblastic origin.

To investigate the presence of PCPE and CT-PCPE in breast tumor cells, medium conditioned by MCF 10A, MCF 7, MDA MB-468, MDA MB-231, HS578T, and a normal fibroblast cell line (BUD 8) were analyzed by reverse zymography and immunoblot to PCPE. Results of this comparison showed that as breast tumor epithelial cells become more aggressive and invasive, not only do they produce more TIMP activity, but they also produce full length PCPE. Furthermore, the more aggressive cell lines processed PCPE to the inhibitor CT-PCPE. As predicted, the normal fibroblast cell line produced full length PCPE. However, there was no evidence of PCPE processing or CT-PCPE inhibitor activity in the medium conditioned by this fibroblast cell line. This suggests that in addition to producing PCPE, the more aggressive breast tumor cells produce proteinases that are capable of processing PCPE to the inhibitor CT-PCPE. It is unclear what role PCPE and CT-PCPE play in the aggressive and invasive potential of breast tumor cells. One hypothesis is that PCPE may be a bifunctional molecule where the inhibitory activity is only reveled by processing. Collagen deposition may be increased by the dual action of PCPE activity coupled with the release of a polypeptide (CT-PCPE) that inhibits the activity of ECM degrading enzymes. This may lead to an increase in ECM components that cause fibrosis associated with some breast tumors.

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LOW RISK OF CANCER RB MUTANTS INTERACT WITH A NUMBER OF CELLULAR PROTEINS

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The tumor suppression effects of retinoblastoma protein (pRB) have been hypothesized to be due to its ability to inhibit transcription factor E2F and cause arrest in cell cycle. However, in addition to regulating the cell cycle, pRB also regulates other processes that affect cell proliferation, such as differentiation. Indeed, the reason why mutations in the pRB pathway are so common in breast cancers may be because pRB is an integrator of diverse signals that ultimately govern the ability of breast cells to grow in a proper controlled manner. Among pRB mutants, our laboratory previously identified several mutants associated with a low risk of cancer that, unlike the high risk mutants, retained the ability to promote differentiation. We used the low risk pRB mutants to specifically track the pRB interacting proteins important for differentiation. In the present study, we applied both biochemical and genetical approaches. As a result of these screenings, a number of proteins were isolated with the low risk of cancer pRB mutants, which all had consistently higher binding to these proteins than to the E2F-1. RB-Binding Protein 2 (RBP2), was investigated in greater details. RBP2 protein complexes reside on certain sites in chromatin in differentiation-dependent manner. One of the models is that the pRB interacting proteins act at a differentiation checkpoint by regulating cell-cycle progression and tissue-specific gene expression. These proteins may, themselves, be dysregulated in breast cancers, even in cells with normal pRB. The closest human homolog of RBP2, PLU-1, has been shown to be unregulated in breast cancers with the highest expression being seen in the invasive component. If so, these proteins are potentially amenable to pharmacological manipulation.

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PROTEIN INTERACTIONS MONITORED VIA BETA-LACTAMASE COMPLEMENTATION. POTENTIAL APPLICATION TO BREAST CANCER

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Previously, we developed a system to study protein-protein interactions based on beta-galactosidase complementation. This system utilizes two beta-galactosidase truncation mutants which have very little enzymatic activity when co-expressed. However when these mutants are fused to two proteins that dimerize, the dimerization of the proteins forces the interaction of the beta-galactosidase mutants thus regenerating enzyme activity. The advantages of this approach are direct signal generation from the interaction, robust signal to noise ratio, and easy adaptation to automated high throughput screening.

Of particular relevance to breast cancer, we have used this system to monitor the dimerization of the epidermal growth factor receptor (EGFR). Although beta-galactosidase complementation is an excellent tool to study protein interactions, the system can be limited by the size of the mutant subunits (>100 kDa), or the fact that beta-galactosidase is only active as a homotetramer, necessitating the formation of higher order structures. Therefore we developed a similar system based on the beta-lactamase enzyme which retains the positive attributes of the beta-galactosidase enzyme complementation system and alleviates many of the aforementioned difficulties. We tested whether it was possible to use the beta-lactamase complementation system in mammalian cells to monitor an inducible protein interaction. We fused two complementing beta-lactamase fragments to FKBP12 and FRB. FKBP12 binds FRB only in the presence of the small molecule rapamycin. Induction of protein dimerization by addition of rapamycin in vivo to cells expressing the fusion proteins resulted in a 10-100 fold increase in cleaved beta-lactamase substrate, monitored either by immunofluorescence or flow cytometry. The reaction is 70% maximal within fifteen minutes and remarkably, shows little or no background activity in the absence of protein dimerization. In conclusion, we have generated a robust system to monitor protein-protein interactions which has specific advantages in comparison to the beta-galactosidase complementation system and should prove useful for high-throughput drug screening applications.

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ROLE OF LIPID RAFTS IN BREAST CANCER

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(a) Introduction: Purpose of Study. Lipid rafts are microdomains in the cell surface, enriched in cholesterol and sphingolipids, in which lipids are packed together unusually tightly. A number of signal transduction proteins are concentrated in rafts in many cells, and must localize to rafts in order to function. Rafts are also likely to be important in signaling in breast cancer cells as well. One specific example of how this might occur in breast cancer involves uPAR, the urokinase-type plasminogen activator receptor, which is a key player in metastasis of breast cancer cells. uPAR is a member of the GPI-anchored protein family, all of which are localized to rafts. To test these ideas, we are examining the effects of disrupting rafts on signaling in breast cancer cells.

(b) Description of Experimental Procedures. All of our experiments are performed in cultured cell lines. In some cases, early experiments have been performed in fibroblasts or other cells. Results from these experiments are validated in cultured breast cancer cell lines, focusing on the MCF7 line. Cells in culture are treated with various raft disrupting agents and the effects are examined by polyacrylamide gel electrophoresis and Western blotting to visualize specific proteins.

(c) Summary of Results to Date. We found that disrupting rafts by removing cholesterol with the cholesterol-binding agent methyl beta cyclodextrin (MBCD) had profound effects on cells. We found hyper-stimulation of the downstream signaling protein MAP kinase. We also tested the effect of sphingomyelinase on raft disruption. This enzyme degrades sphingomyelin, an important lipid component of rafts. We found that cellular levels of sphingomyelin were reduced by at least 80%. Surprisingly, rafts were not destroyed, and a large number of proteins still associated with low-density detergent-resistant membranes.

(d) Conclusions. We conclude that MBCD effectively disrupts rafts in breast cancer cells, and that raft disruption leads to hyper-stimulation of at least some signaling pathways. This suggests that normal controls on these pathways, which prevent aberrant signaling, require the presence of rafts. We also conclude that sphingomyelinase treatment does not effectively disrupt rafts.

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THE ATOMIC STRUCTURE OF THE HUMAN NUCLEAR CAP BINDING COMPLEX (CBC) REVEALS DOMAIN CLOSURE UPON METHYLATED CAP STRUCTURE BINDING

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This project involves structural studies of a nuclear target for the EGF receptor, and the related Neu/ErbB2 tyrosine kinase, named the CBC for RNA-capped binding protein complex. The CBC consists of two subunits, CBP20 (Mr 18 kDa) and CBP80 (Mr 90 kda), and undergoes a growth factor (EGF, heregulin)-dependent binding of RNAs transcribed by the RNA polymerase II. While, EGF stimulates CBC activity, it is most strongly stimulated by heregulin, an activator of the Neu/ErbB2 tyrosine kinase, and appears to be constitutive in breast cancer where Neu/ErbB2 expression is high. Biochemical and genetic experiments have shown important roles for CBC in mRNA physiology including splicing, nuclear export, 3' end processing, translation initiation and nonsense mediated decay (NMD). We have solved the atomic structure of CBC alone and in complex with the cap analog m7GpppG at 2.6 and 2.1 angstroms, respectively. CBP80 is a super-helical structure and consists of three domains connected by two long linkers. CBP20 conforms to a classical ribonucleotide binding (RNP) domain and reveals a new role for RNP proteins namely, methylated cap structure binding. CBP20 binds the methylated guanosine base via pi-pi stacking interactions and a hydrogen bond network that resembles Watson-Crick pairing between guanosine and cytosine. Structure comparisons between the free and bound forms of CBP20 show that domain closure about two hinges promotes cap structure binding. CBP80 plays a crucial role in domain motion by providing structural rigidity to one of these hinges and could represents a novel regulatory mechanism in RNA binding proteins. Furthermore, the ability of CBP80 to interact with several proteins involved in mRNA processing coupled to its role in the regulation of CBP20's binding affinity for the capped structure could represent a mechanism to ensure mRNA processing. Further biochemical, genetic and crystallographic studies are needed to fully understand the role of CBC in mRNA biology and to discern the higher level of regulation brought about by signaling events via EGF-R or Neu/Erb2. By participating in most of the steps that regulate mRNA metabolism the CBC could play an important role in the regulation of protein expression in the cell.

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THE NONCLASSICAL SECRETION OF THE GROWTH FACTOR THIOREDOXIN

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Thioredoxin is a small cytosolic protein (12 kDa) that participates in cellular redox reactions through two cysteine residues in its active site that act as electron donors. The normal functions of intracellular thioredoxin include activating transcription factors, assisting in protein folding, and maintaining redox status. Thioredoxin can also be found extracellularly where it stimulates anti-parasitic activity of eosinophils or aids in the establishment of the placenta. Various tumor cells including breast cancer cells secrete thioredoxin that acts as a growth stimulant. The over-expression of thioredoxin in MCF-7 breast cancer cells results in increased secretion of thioredoxin, more rapid proliferation of cells, and the formation of larger tumors in mice. Mutations in the active site of thioredoxin completely abolish its growth stimulating effects. Thioredoxin is secreted via a non-classical pathway which is poorly understood. However, this pathway that does not appear to use the endoplasmic reticulum and Golgi apparatus since export of thioredoxin is not inhibited by brefeldin A.

We are studying the secretion of thioredoxin from mammalian cells to identify regions in the protein that target it for export and the cellular proteins involved in this process. An understanding of this pathway may make it feasible to inhibit thioredoxin export and thereby slow the growth of tumors. To study the targeting and export of human thioredoxin from mammalian cells, we used transiently-transfected Chinese hamster ovary cells since they lack cross-reactive endogenous thioredoxin. These cells synthesize and secrete human thioredoxin protein in a brefeldin A-insensitive manner just as is observed for MCF-7 and HT-29 cells (derived from colon cancer) indicating that the secretion occurs via a non-classical pathway. The kinetics of thioredoxin secretion is slow with up to 50% of the protein released into the medium over 8 hr. Secretion is inhibited by low temperature and factors present in serum. Release of thioredoxin does not appear to involve membrane blebbing as has been described for galectin-3. A mutation of one cysteine residue in the active site does not impair secretion of thioredoxin from transfected Chinese hamster ovary cells. Similarly, mutation of the cysteine of the regulatory homodimerization site also had no significant effect on thioredoxin secretion. Thus, secretion of thioredoxin is independent of the redox state of the protein. We are continuing to study the targeting signal and cellular proteins involved in the non-classical secretion of thioredoxin in Chinese hamster ovary and MCF-7 breast cancer cells.

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THE BH3 MOTIF OF PRO-APOPTOTIC BID POSSESSES EXTENDED SPECIFICITY BOUND TO BCL-X_L

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Programmed cell death is a highly evolutionarily conserved biological process critical for development and homeostasis in multicellular organisms. The study of complex signaling pathways of programmed cell death has led to the identification of a large number of molecules involved in regulating apoptotic death, or promoting cell survival. Key features of programmed cell death include, of several mechanisms, a cascade of proteases tightly controlled by apoptotic signaling pathways. As part of a critical apoptotic pathway regulating a critical checkpoint in mitochondria, the Bcl-2 family of proteins comprises both anti- and pro-apoptotic regulators. Various approaches to modifying the Bcl family's activities are proposed as potential cancer therapeutic approaches.

Membership in the Bcl-2 family is defined based on homology to at least one of four conserved sequence motifs known as Bcl-2 homology motifs, BH1 to BH4 (sometimes referred to as domains). Most anti-apoptosis family members have BH1 and BH2 motifs and many have all four BH motifs. In contrast, many of the pro-apoptotic family members (Bid, Bad, Bik, Bim, Blk, Hrk) only possess the conserved BH3 motif. The ratio of anti- and pro-apoptotic molecules apparently determines whether a cell will respond to a proximal apoptotic stimulus. This competition is mediated, at least in part, by competitive dimerization between anti and pro-apoptotic pairs. However, other roles including self association into ion transporters in membranes, and specific association with membrane associated receptors (protein or lipid) in organelle membranes have been also considered.

An important sub-family within the Bcl-2 family consists of pro-apoptotic molecules with homology only in the BH3 region. The BH3-only class of cell death effectors are conserved components of a central death pathway. The Bcl-2 family member Bid belongs to the BH3-only class of pro-apoptotic killers. Like other members of this class it possesses a BH3 motif for its dimerization and pro-apoptotic activity. Caspase-8 mediates the cleavage of an inactive 23 kDa cytosolic Bid to produce a truncated 15 kDa fragment (referred to as tBid) that translocates to the mitochondria causing cytochrome c release, activation of caspases and the final commitment to cell death. While developing structural approaches to the tBid/Bcl-x_L complex, we determined first the structure of the BH3 motif of tBid bound to Bcl-x_L which has significantly greater contact area than the previously suggested BH3 motif binding, with possible implications for the design of small molecular activators of the Bcl pathway.

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CLONING AND CHARACTERIZATION OF CLAN, A NOVEL CED-4 HOMOLOG THAT REGULATES CASPASE-1 ACTIVITY

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Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1B) is a cytokine which has been found to be expressed in breast cancer cells and may be associated with more aggressive and invasive breast tumors. Through a bioinformatics approach, we identified a novel CARD protein which also contained a nucleotide binding domain (NACHT) and a region of leucine-rich repeats (LRR). This protein, which we named CLAN (CARD, LRR, And NACHT-containing protein), is classified as a member of the CED-4-like protein family which also includes the homologous human proteins Apaf-1, Nod1 and Nod2. By searching the HTGST database of human genomic DNA sequence data using the amino acid sequence of cIAP1 as a query, the previously unrecognized and unannotated CLAN locus was identified on chromosome 2p21-p22. Here we report the cloning and functional characterization of CLAN and several of its alternatively spliced isoforms. CLAN was found to be expressed in several breast cancer cell lines as well as in monocytes by RT-PCR. To determine the physiologic function of CLAN, its CARD was epitope-tagged and co-expressed in HEK293T cells with other CARD-containing proteins. Co-immunoprecipitation studies revealed that the CARD of CLAN associated with the CARD of several other proteins including caspase-1, Nod2, and NAC. Caspase-1 is a cysteine protease known to have a bearing on both apoptosis and cytokine production, depending on the cell type. When assayed using an IL-1B ELISA, CLAN was found to induce the activation of caspase-1, presumably through the induced proximity mechanism of caspase activation. Although many new CARD proteins have recently been found to bind and negatively regulate caspase-1, CLAN represents one of the only CARD proteins known to activate a caspase. Through its interactions with other CARD-containing proteins, CLAN may regulate the survival of breast cancer cells and could be utilized as a novel anti-tumor target or diagnostic/prognostic biomarker.

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PROTEOLYTIC INSTABILITY IN BREAST CANCER CELLS

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Protein degradation, in concert with protein synthesis, governs the proper execution of metabolic processes in the cell. The large, intracellular proteases like anti-cancer drug target proteasome, a novel protease multicorn, tripeptidyl peptidase II (TPPII) or leucine aminopeptidase (LAP) play a key but poorly understood role among all proteolytic enzymes due to their diverse functions. The proteasome actions are essential for cell cycle regulation, turnover of transcription factors and antigen processing. Inhibition of proteasome leads to cell death and is utilized to kill tumor cells. However, there are strong indications that the other proteases may also constitute valuable anti-cancer drug targets. The postulated duties of multicorn include degradation of cell cycle related factors and, together with TPPII and LAP, further processing of antigenic peptides produced by proteasome. The collaboration of proteasome and other large proteases suggests the existence of a net of functional relationships between the executors of controlled proteolysis.

We show here that proteolytic instability, which manifests in changing the equilibrium between the activities of large cytosolic proteases is one of the signs of neoplastic transformation in human breast cancer MCF7 cells, as compared with non-cancerous MCF10A cells. To demonstrate the proteolytic instability we quantified the amount of enzymes with Western blotting, and determined their proteolytic activities with high-throughput methods using specific fluorogenic peptide substrates.

In the functional proteomics fashion we found prominent changes on both the functional level of activities and specificities of the enzymes, and the structural level of subunit composition, modifications and subcellular distribution. Specifically, we found that there is markedly less proteasomes in nuclei of the cancerous than control cells, and nuclei of cancer and control cells have dramatically different pattern of subunits of a natural proteasome activator. Since proteasome, multicorn, TPPII and LAP all take part in antigen processing, the changes may impair removal of transformed cells by the organism. Probing the role of large proteases in maintaining the proper advance of cell cycle we discovered that the predominantly cytosolic multicorn can be found in the nucleus, but this residence is much more pronounced in cancerous than in control cells. Interestingly, both nuclei and cytosol of cancer cells contain mostly overphosphorylated, unstable forms of the protease. We postulate that the nuclear localization of multicorn holds the key for dissecting the role of this protease in cell cycle progression.

Our findings show a fragment of potentially critical interactions between parts of the controlled proteolysis machinery. This web of interactions should be taken into account when anti-cancer drugs target single components of the whole system.

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IDENTIFYING A NOVEL COAT PROTEIN THAT MEDIATES THE FORMATION OF ARF6-REGULATED VESICLES

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Tumors metastasize by commandeering mechanisms of cell migration, a process that requires two major intracellular events – actin rearrangement and endocytic transport – for the formation of surface protrusions that mediate motility. The small GTPase ADP-ribosylation factor 6 (ARF6) has been shown to regulate endocytic recycling, and overexpression of a dominant negative mutant (ARF6-T27N) induces the accumulation of coated vesicles. We have purified these vesicles to homogeneity and shown that they contain a novel coat complex and contain cargo proteins that undergo endocytic recycling (Peters, PJ, Gao, M, et al, (2001) *Traffic* 2: 885-895). Currently, our goal is to identify the novel coat complex.

One approach that we have taken was to scale-up our purification protocol. However, pilot experiments revealed that the final step needed to purify the sample from 50% to near 100% also led to markedly reduced yield. Thus, as an alternative strategy, we first performed two-dimensional (2D) gel electrophoresis on highly purified vesicles that had been radioiodinated on the cytoplasmic face, as the most abundant proteins by this approach would be predicted to be candidate coat proteins. Subsequently, 2D gel was also obtained on the enriched fraction (50% pure), reasoning that proteins in this profile that colocalized with those on the highly purified iodinated sample would correspond to the candidate coat proteins. By this match-up approach, we could not detect correspondence with the most abundant proteins, suggesting that our purification procedure might have been too harsh to retain the coat complex on purified vesicles.

In considering an alternate strategy, we are taking advantage of another finding that the v-SNARE, cellubrevin, is a cargo protein of the purified vesicles. While the main role of v-SNAREs is to mediate vesicle docking/fusion, they have also been shown to participate in coat recruitment, by serving as docking sites for coat proteins. Thus, we have generated a GST fusion protein that contains the cytoplasmic domain of cellubrevin and plan to use a pulldown technique to extract the novel coat complex from cytosol, as coat proteins have been shown to exist as intact complexes in the cytosol.

The eventual identification of a novel coat complex regulated by ARF6 will provide a better molecular understanding of the recycling pathway that mediate cell migration. This knowledge provides additional biochemical targets for the future rational design of therapeutic intervention against tumor metastasis.

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PHOTOAFFINITY LABELING OF THE INSULIN-LIKE GROWTH FACTOR (IGF)-BINDING DOMAIN ON IGF-BINDING PROTEIN-3

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The IGF system plays an important role in cell growth and development. In this system, the IGFBPs serve as natural antagonists of IGF action through sequestration of IGF from the IGF-1 receptor (IGF-1R). Enhanced activity of the IGFs has been implicated in increased tumorigenesis, thus blockade of IGF action may be clinically significant. At present, the binding interaction between IGF-1 and the IGFBPs is not clearly understood. To this end, we have synthesized IGF photoprobes for use in cross-linking studies with the IGFBPs to identify potential sites of contact between IGF-1 and the IGFBPs. IGF-1 was derivatized at the Glycine-1 alpha-amino group with a biotin-containing azidobenzoyl group (SBED); this residue is located within the known IGFBP binding domain on IGF-1. Competition binding analyses show that the addition of the azidobenzoyl group does not affect the affinity of the IGF-1 photoprobe for IGFBP-2 or -3. Previous work in our lab using this IGF-1 photoprobe has identified the carboxy-terminus of IGFBP-2 as contacting the IGFBP-binding domain on IGF-1 (*JBC* 276:2880-2889, 2001). We propose to extend these photoaffinity labeling studies to IGFBP-3, the predominant binding protein in human serum. Recent studies have suggested that IGF-1 interacts with the amino-terminus of IGFBP-3. Preliminary experiments show that the Gly-1 IGF-1 photoprobe crosslinks to IGFBP-3. The IGF-1--IGFBP-3 complex was trypsin digested and biotinylated fragments were purified by avidin-agarose chromotography. The site of contact between the IGF photoprobe and IGFBP-3 is currently being analyzed by mass spectrometry. Furthermore, we are repeating these experiments using an IGF-1 photoprobe that is derivatized with SBED or HSAB (azidobenzoyl group alone) at Lysine-27, a residue located near the IGF-1R binding domain. These studies will determine if IGFBP-2 and IGFBP-3 contact the IGF-1R binding domain on IGF-1, thus suggesting a mechanism for IGFBP inhibition of IGF-1 access to the receptor. To further corroborate these findings, carboxy-terminal fragments of IGFBP-2 will be expressed and tested for their affinity for IGF-1. The information obtained from these studies will aid in the clarification of the role of the binding proteins in IGF-mediated tumorigenicity, which will further allow for the rational design of IGF antagonists that are based on the structure and function of the IGFBPs.

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BETA-CATENIN REGULATION IN BREAST CANCER

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B-catenin functions in cadherin mediated cell adhesion, but it can also act independently as a signaling intermediate in oncogenic pathways. Increases in cytoplasmic B-catenin levels are common in several cancer cell lines implying that it is acting as a signal to regulate proliferation. When B-catenin is active, it forms a complex in the nucleus and acts as a transcriptional activator to regulate genes that stimulate proliferation or antagonize apoptosis. Dysregulation of the stringent control over B-catenin protein stability leads to aberrantly elevated levels of B-catenin and subsequent oncogenic signals. The half-life of B-catenin is very short and its degradation is tightly regulated. Disruption of the machinery that normally leads to B-catenin degradation is the major cause of cytoplasmic accumulation leading to uncontrolled proliferation. With this in mind the stability of the protein is commonly viewed as the dominant aspect of B-catenin's regulation. Since B-catenin signaling is known to have an oncogenic effect on cells, it is important to understand the specifics of how the protein is regulated and degraded if new therapies are to be developed. This study investigates the mechanism of degradation and attempts to identify residues in the protein involved in the degradation process. Here we have identified specific residues in the N-terminus of B-catenin important for it's stability, tafficking and regulation.

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ANALYSIS OF THE RAD52/RPA COMPLEX: EVIDENCE FOR CROSSTALK BETWEEN RPA32, RPA70, RAD52, AND SSDNA

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The eukaryotic single-stranded DNA-binding protein, replication protein A (RPA), is essential for DNA replication, and plays important roles in DNA repair and DNA recombination. Rad52 and RPA, along with other members of the Rad52 epistasis group of genes, repair double-stranded DNA breaks (DSBs). Two repair pathways involve RPA and Rad52, homologous recombination and single-strand annealing. Two binding sites for Rad52 have been identified on RPA. They include the previously identified C-terminal domain (CTD) of RPA32 (residues 224 to 271) and the newly identified domain containing residues 169 to 326 of RPA70. A region on Rad52, that includes residues 218 to 303, binds RPA70 as well as RPA32. The N-terminal region of RPA32 does not appear to play a role in the formation of the RPA:Rad52 complex. It appears that the RPA32CTD can substitute for RPA70 in binding Rad52. Sequence homology between RPA32 and RPA70 was used to identify a putative Rad52 binding site on RPA70 that is located near DNA binding domains A and B. Rad52 binding to RPA increases ssDNA affinity significantly. Mutations in DBD-D on RPA32 show that this domain is primarily responsible for the ssDNA binding enhancement. RPA binding to Rad52 inhibits the higher-order self-association of Rad52 rings. Implications for these results for the "hand-off" mechanism between protein-protein partners, including Rad51, in homologous recombination and single-strand annealing are discussed.

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IN VITRO SYSTEM FOR FILOPODIA FORMATION

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The acquisition of a motile phenotype is an important step in the development of tumors and ultimately metastasis. Ultrastructural analysis indicates that transformed breast cells differ from control cells by the development of long filopodia, spike-like extensions of the cell containing a bundle of actin filaments. The actin bundling protein, fascin has a role in construction of filopodia which leads to increased cell motility.

We report here the initial development of an in vitro system for filopodia formation. The brain extract when highly diluted gave rise to star-like actin assemblies which consisted of radial actin bundles emerging from an actin cloud proximal to the bead. Coating of the beads with Arp2/3-activating proteins was required for star formation. Time-lapse microscopy showed that thin actin bundles arose from the actin cloud followed by elongation and coalescence into thicker bundles. The efficient bundling of actin filaments by fascin into parallel bundles within filopodia may promote linear extension and stiffening of filopodia. Immunofluorescence of stars showed that fascin is present along the whole length of actin bundles. Addition of capping protein to extracts blocked bundle formation, but allowed for continuous growth of actin clouds. This suggests that star formation results from low levels of barbed end capping. Pulse-labeling showed that actin incorporation was at the bead surface and at the tips of actin bundles. EM demonstrated that clouds consisted of a dendritic actin network whereas bundles consisted of long unbranched filaments of uniform polarity with barbed ends pointing away from the bead as determined by myosin S1 fragment decoration. The actin filaments of star-bundles share with filopodia in living cells the properties of being long, unbranched, parallel and of uniform polarity. Both structures grow at their barbed ends and require fascin for their formation. As the presence of filopodia can serve as a hallmark of breast cancer cells and the beginning of metastasis, it is important to understand the mechanism of filopodia formation. Therefore, we propose that actin stars may serve as a model for filopodia formation in vitro. Molecular players in this process could serve as novel targets for the treatment of cancer.

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BREAST EPITHELIAL CELL MEMBRANE TRANSPORTERS MAY DETERMINE EXPOSURE OF CELLS TO ANTIOXIDANTS AND CARCINOGENS

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Hope for prevention of breast cancer arises from international breast cancer rate variation, which suggests potentially modifiable environmental determinants of the disease. We used immunocytochemical and Western blot methods to localize two membrane xenobiotic transporters, MRP1 and MRP2 of human breast epithelial cells. Other studies have shown that MRP2 is located at the apical membrane of epithelial cells and excretes anionic hydrophobic substrates, such as 17 beta-estrogen glucuronide, antioxidants, and toxic molecules into lumens of the kidney, liver, and gut. In isolated and tissue cultured breast ductules, MRP2 is located predominantly in the apical region of luminal epithelial cells and thus might bioconcentrate toxic substrates into sealed, non-flushed lumens. In a comparison epithelium, Caco2 cells in culture, MRP2 is localized at the apical membrane only and is detected as bands at 60 and 190 kDa in blots, but in breast tissue cultures MRP2 is detected only at 60 kDa. In other epithelia, MRP1 transports anionic hydrophobic molecules across the basolateral side of epithelial cells into the connective tissue. In isolated and tissue cultured breast ductules, MRP1 is localized in the cytoplasm, as is typical for numerous epithelial cells and is detected as a band at 190 kDa. Thus, overall transport of toxins to, and bioconcentration in, breast ductule lumens may be determined by a balance between these two transporters. Human nipple aspirate fluid and breast milk contain a number of MRP2 substrates, suggesting that breast epithelial cells have both the metabolic pathways (P450 and conjugating enzymes) and transporters, such as MRP2, to metabolize certain hydrophobic molecules which enter the basal side of the cell from connective tissue. A full understanding of these transport and metabolic pathways should lead to specific nutrition recommendations for breast cancer prevention.

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ROLE OF NOVEL ROS-GENERATING ENZYMES IN BREAST CANCER DEVELOPMENT AND PROGRESSION

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Certain human carcinoma cell lines produce reactive oxygen species (ROS) constitutively. This affords cancer cells potentially with an advantage in cell proliferation, migration and invasion of surrounding tissues by degrading extracellular matrix and thereby increasing their motility. The basis for generation or upregulation of ROS in cancer cells is unknown. A novel class of ROS-generating flavoenzymes with homology to a subunit of the NADPH oxidase component cytochrome b_{558} has been identified recently. One member of this family, called Nox1, has been implicated in tumor development in mice. Overall, the regulation and cellular functions of Nox1 and three other related Nox isoforms are still unknown.

We have characterized several breast cancer cells for Nox isoform expression as well as the presence of several NADPH oxidase components. Nox 4 and Nox 5 have been detected by RT-PCR, while the cytochrome b subunit gp91 (now Nox 2) and some other oxidase proteins were absent. Various inhibitors directed against potential ROS-producing enzymes have been tested in breast cancer cells, but were predominantly ineffective. ROS generation was dependent on several cellular parameters such as adhesion, nutrition and cell density.

To investigate the effects of Nox isoforms in a less transformed, more normal cellular phenotype, we established stable HEK293 and Cos cell lines expressing Nox 4 and Nox5. Nox 4 cells showed constitutive activity resulting in intracellular ROS production and hydrogen peroxide release, while Nox5 activity could not be detected. Dominant negative Rac1 or Ras, two signaling molecules intimately involved in ROS generation in fibroblasts, did not affect H_2O_2 release. We tested the ability of Nox 4 cells to migrate in *in vitro* migration assays and determined a slight decrease in motility when compared to control cells. This effect could be caused by differences in adhesion. Initial results suggest that active Nox 4 may alter the attachment on extracellular matrix. The ability to decrease attachment is an important element for dissemination of cancer cells and their ability to metastasize. Nox proteins and their products (ROS) may contribute to the neoplastic phenotype, and the delineation of their cellular functions may have implications for the control of metastatic events in breast cancer.

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HSOF1, A U3 SNORNP COMPONENT, IS ASSOCIATED WITH CYTOPLASMIC RIBOSOMES

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During transformation ribosome biogenesis increases concurrently with the size and number of nucleoli. Ribosome biogenesis is closely regulated and requires many factors that act in pre-ribosomal RNA (pre-rRNA) transcription and processing and in preribosomal particle assembly and export. The U3 small nucleolar ribonucleoprotein (snoRNP) is a complex that directs specific cleavages of pre-rRNA and is involved in 40S preribosomal particle assembly. The U3 snoRNP consists of the U3 small nucleolar RNA (snoRNA) and a number of associated proteins, including Sof1 and fibrillarin. The exact roles of these proteins in the U3 snoRNP are unclear.

Our laboratory has cloned human Sof1 (hSof1). hSof1 is the 48 kDa homolog of yeast Sof1 (ySof1) and shares the similar domain structure of seven WD40 repeats and unique amino- and carboxy-termini. Depletion of ySof1 inhibits pre-rRNA processing, consistent with a functional role in the U3 snoRNP.

Our research focuses on characterizing the role of hSof1 in ribosome biogenesis by examining its localization and dynamics in live cells and its interactions. hSof1, like fibrillarin, localizes to both the nucleolus and nucleoplasm. However, unlike fibrillarin, hSof1 is not limited to the dense fibrillar components of nucleoli and responds differently to the inhibition of the transcription of pre-rRNA. In addition, hSof1-GFP also exhibits a higher nuclear mobility than fibrillarin-GFP and is a nucleocytoplasmic shuttling protein. Recent experiments have shown that hSof1p is associated with cytoplasmic ribosomal subunits, indicating that the protein may remain associated with preribosomal particles when they traverse from the nucleolus to the cytoplasm. We are currently mapping the domains of hSof1, attempting functional disruption, and plan to determine its tertiary structure. These experiments will clarify the role of Sof1 within the U3 snoRNP and any further roles in ribosome biogenesis. These findings will contribute to our understanding of ribosome biogenesis as a whole and may expose regulatory points within the process that could be utilized as anti-cancer drug targets.

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MATRIPTASE, A NOVEL TYPE-2 MEMBRANE SERINE PROTEASE, IS AN ACTIVATOR OF HEPATOCYTE GROWTH FACTOR AND PRO-UPA

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Matriptase is an epithelial-derived, type 2, integral membrane, serine protease. It contains an N-terminal putative transmembrane sequence, followed by multiple LDL and CUB repeats, and the C-terminal protease domain. The enzyme was originally identified in human breast cancer cells, it was then found expressed in breast epithelial cells, in both estrogen positive and negative breast carcinomas, and in other human carcinomas including ovarian, endometrial, and colon. To further understand its role in breast cancer, we have investigated its substrate specificity. By using fluorescence-labeled peptide substrates in the enzyme activity assay, we demonstrated in this report that active matriptase purified from the human milk specifically cleaves synthetic peptides after an Arg or Lys residue, and prefers small side-chain amino acids, such as Ala and Gly, at the P2 site. We further demonstrated that matriptase can convert single chain hepatocyte growth factor/scattering factor precursor to its active form that can induce scatter of Madin-Darby canine kidney epithelial cells and can activate c-Met tyrosin phosphorylation in A549 human lung carcinoma cells. Matriptase is also an activator of pro-uPA. It cleaves and converts pro-uPA to an enzymatically active two-chain uPA. These results further support our hypothesis that matriptase acts as an upstream activator in metastasis and cancer invasion by interacting with and recruiting various factors to the site of contact between cancer and stromal cells; and by degrading or processing a broad range of substrates. Our studies demonstrate a target for prevention of cancer invasion and metastasis.

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SIGNALING EVENTS IN THE SPATIAL REGULATION OF CAMKIV ACTIVITY

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In situ hybridization studies demonstrate that CaMKIV, a multifunctional calcium-calmodulin dependent serine/threonine protein kinase, is regionally expressed during mouse development. Further, evidence from the CaMKIV knockout mouse suggests that it is involved in cellular differentiation of tissues such as the breast and ovary. To better understand the role of CaMKIV in cellular differentiation, we examined its localization in cultured cells. We divided the kinase into functional domains, fused each to the yellow fluorescent protein, YFP, and visualized them by fluorescence microscopy. The catalytic domain enters the nucleus and the autoregulatory carboxyl-terminal domain remains in the cytoplasm. These observations demonstrate that the information necessary for CaMKIV to enter the nucleus resides within the catalytic domain. To address whether catalytic activity affects the localization of the full-length enzyme, we made point mutations in the small ATP binding domain, Lys 71 to Met, and in the large protein substrate binding domain, Glu 207 to Lys, of the kinase. Each mutation resulted expression of a full-length, inactive, protein kinase that remained in the cytoplasm. Based on these observations, we evaluated the other steps of CaMKIV activation; Ca2+/CaM binding, removal of the autoinhibitory domain, and activation-loop phosphorylation, in relation to nuclear entry. Mutating Leu 322, the hydrophobic residue predicted to anchor Ca2+/CaM to CaMKIV, or Leu 314 to remove the autoinhibitory domain from the catalytic cleft, did not change the nuclear localization of the enzyme. To determine whether activation-loop phosphorylation was required for nuclear entry, we mutated the activation-loop Thr 196 to Ala. Because YFP-CaMKIV-T196A enters the nucleus, we propose that nuclear entry of CaMKIV requires catalytic activity, but is a phospho-activation-loop-independent process.

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EXPRESSION OF THE HEPATOCYTE GROWTH FACTOR ACTIVATOR INHIBITOR (HAI-1) IN MDA MB-435 HUMAN BREAST CANCER CELLS REDUCES GROWTH IN VITRO AND IN VIVO

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We have over-expressed the hepatocyte growth factor activator inhibitor-1 (HAI-1) in a human breast cancer cell line, MDA-MB-435. HAI-1 is a Kunitz-domain containing serine protease inhibitor that has been shown to inhibit the activity of the hepatocyte growth factor activator (HGFA) and matriptase (also termed ST14, MT-SP1 and TADG-15). Both of these serine proteases have been shown to cleave and activate the growth/motility factor hepatocyte growth factor (HGF). In addition, matriptase has been shown to cleave and activate the serine protease urokinase plasminogen activator (uPA) and the protease-activated receptor-2 (PAR-2). Stable expression of HAI-1 in the MDA-MB-435 breast cancer cell line induced a profound reduction in cell growth in vitro and in tumor xenografts in vivo. Despite the dramatic reduction in cell proliferation, HAI-1 expression in MDA MB-435 tumor xenografts did not reduce the incidence of tumor micro-metastases in nude mice. This indicates that HAI-1 affects the proliferation of MDA MB-435 cells, but not their metastatic potential in nude mice. Moderate in vitro changes were also observed in cellular morphology, but no changes were seen in cellular motility. The growth reduction induced by HAI-1 expression in MDA MB-435 cells may be explained by the inhibition of serine proteases such as HGFA, or by the inhibition of as-yet uncharacterized serine proteases.

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STRUCTURE AND FUNCTION OF THE MAMMARY TUMOR PROTEIN (MAT8) IN A LIPID ENVIRONMENT

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The mammary tumor protein (Mat8) is an ion channel expressed in human breast tumors, breast tumor cell lines, and prostate cancer cell lines. Its channel activity provides insight to its function and potential as a drug receptor, and suggests a possible role in transepithelial transport, breast tissue homeostasis, cell proliferation and cell cycle regulation. In order to understand the function of Mat8 it is essential to correlate its structure with its channel activity and that is the primary goal of this research. The structure of Mat8 is also the starting point for the rational design and screening of specific anti-cancer drugs. The aims of the research are: (1) to determine the structure of Mat8 in membrane environments, (2) to characterize its ion channel activity, and (3) to identify molecules that interfere with its activity.

We have worked out procedures for the expression and purification of recombinant Mat8. Briefly, the Mat8 gene has been synthesized and cloned in a fusion protein *E. coli* vector developed for membrane protein expression and purification. The fusion protein forms inclusion bodies and is expressed at levels up to 20% of the total cellular protein in *E. coli* strain BL21(DE3). The inclusion bodies are separated from lysed cells, and isolated by Nickel column chromatography. Mat8 is cleaved from the fusion partner using cyanogen bromide and then purified by size exclusion chromatography, followed by reverse phase chromatography. The structure of Mat8 is being determined in membrane environments, using solid-state NMR spectroscopy of oriented lipid bilayers, and solution NMR spectroscopy of lipid micelles. We have obtained NMR spectra in lipids that give the initial view of the protein structure in the membrane. The ion channel activity is being characterized using concentrative ion uptake assays and ion channel recordings of Mat8 in lipid bilayers.

The three dimensional structure of Mat8 will bring important insight to critical events in breast tumorigenesis, and will provide a tumor-specific target for anti-cancer drugs. It is anticipated that the structure of Mat8 will shed light on the role of Mat8 as an ion channel in breast cancer, and pave the way for therapeutic approaches to breast cancer.

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TOWARDS STRUCTURAL STUDIES OF THE AIB1 (AMPLIFIED IN BREAST CANCER-1) PROTEIN

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The long arm of chromosome 20 encodes a gene termed AIB1 (amplified in breast cancer-1) that is amplified in 10% of primary breast cancers, and shows mRNA overexpression in 64% of primary breast tumors. AIB1 is a member of the steroid receptor coactivator (SRC) family of transcriptional regulatory proteins that include SRC-1, AIB1 (also referred to as ACTR, pCIP, TRAM-1 or RAC3), SRC-3, TIF2 and GRIP1. These proteins interact with steroid receptors to promote ligand-dependant transcriptional activation for the regulation of biological processes such as development, reproduction and homeostasis. The SRC proteins contain three highly conserved regions including centrally located LXXLL motifs that mediate direct interactions with liganded nuclear receptors, as well as highly conserved amino- and carboxyl domains. The conserved amino-terminal region contains a bHLH-PAS domain that is found in many transcription factors and is believed to play a role in protein-protein interaction and/or protein dimerization. The conserved carboxyl-terminal region has been shown to harbor histone acetyltransferase (HAT) activity. HAT enzymes play a conserved role in gene activation by acetylating specific lysine residues of histone proteins. In order to obtain new insights into the function of the bHLH-PAS and HAT domains of AIB1 we have been working towards an X-ray crystal structure determination of these domains. We have overexpressed and purified the bHLH-PAS domain of AIB1 to homogeneity and crystallization efforts are in underway. All attempts at preparing recombinant forms of the AIB1 HAT domain have resulted in insoluble protein suggesting that a well-folded AIB1 HAT region may require the cooperation of other protein regions or protein cofactors. We are currently investigating this possibility. The availability of structural information on the bHLH-PAS and HAT domains of AIB1 will provide a more complete understanding of normal AIB1 function and AIB1 malfunction in breast cancer, and will also lay the groundwork for the structure-based design of AIB1-specific inhibitory molecule for therapeutic application.

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CRYSTAL STRUCTURE OF THE MOUSE P53 CORE DNA-BINDING DOMAIN AT 2.7Å RESOLUTION

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Alterations of the p53 tumor suppressor gene are the most common genetic changes found in breast cancer, implicating its central role in the development of the disease. p53 is a sequence-specific DNA binding protein that regulates transcription in response to DNA damage to promote cell cycle arrest or apoptosis. The p53 protein functions in a tetrameric form in vivo and contains four domains including an N-terminal transcriptional activation domain, a C-terminal regulatory domain, a tetramerization domain and a central core DNA-binding domain that is the site of the majority of tumor-derived mutations. Here we report the 2.7Å crystal structure of the mouse p53 core domain. Like the human p53 core domain in complex with DNA, the mouse p53 core domain adopts an immunoglobulin-like beta sandwich architecture with a series of loops and short helices at opposite ends of the beta sandwich; and the majority of tumor-derived p53 mutations map to positions that are predicted to disrupt protein folding or DNA-binding. Comparison of the DNA-bound and DNA-free p53 core domains reveals that while the central beta sandwich architecture remains largely unchanged, a loop region important for DNA binding undergoes significant rearrangement. While this loop region mediates major groove DNA contacts in the DNA-bound structure, it adopts a conformation that is incompatible with DNA-binding in the DNA-free structure. Interestingly, crystals of the DNA-free core domain contain a non-crystallographic trimer with three nearly identical subunit-subunit (dimer) contacts. These dimer contacts align the p53 core domains in a way that is incompatible with simultaneous DNA-binding by both protomers of the dimer. Correlatively, similar dimer contacts are observed in crystals of the human p53 core domain with DNA in which only one of the three p53 protomers in the asymmetric unit cell are specifically bound to DNA. We propose that the p53 core domain dimer that is seen in the crystals described here represents a physiologically relevant inactive form of p53 that must undergo structural rearrangement for sequence-specific DNA binding. These studies suggest a possible drug strategy to lock mutant p53 forms in the active DNA-binding conformation for breast cancer treatment.

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ANTIZYME ACTIVATION IN CHEMOTHERAPY AND CHEMOPREVENTION

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The polyamines are small organic cations that are essential for normal cell physiology and growth. Because of their central role in cell growth and death, the regulatory pathways of the polyamines have been targeted as a means to elucidate the mechanisms of cancer. A small, labile protein, found in higher eukaryotes, called antizyme, plays a critical role in polyamine regulation. Although an extremely important protein in the regulation of polyamines and a likely candidate to mediate polyamine depletion as cancer therapy, very little is known about antizyme form and function.

Antizyme, which was once thought of as a single protein, has recently been found to actually consist of at least four different forms in cells. These forms are distinct in their molecular weight as well as stability and response to osmotic stress. To determine what forms originate from the first start site of AZ-1 and what post-translational modifications occur, antizyme constructs with the second start site mutated were made and transfected into rat hepatoma (HTC) and Chinese hamster ovary (CHO) cells.

ODC-antizyme assays on the mutated antizyme, purified from cells, showed that the mutated antizyme is active in binding to and inhibiting ODC. Cell fractionation experiments showed that the mutated antizyme localizes similarly to wild-type antizyme. Suprisingly, Western blot analysis showed that the mutated antizyme gene, when expressed in cells, produces two proteins distinct in molecular weight. Only one band running at 37 kDa was expected, and when expressed in a cell-free system only one band running at 37 kDa was seen. The second, smaller band found in cells can only be explained by post-translational modification, specifically proteolytic cleavage. Furthermore, the pattern of expression for these two forms is consistent with the two major antizyme forms normally found in cells.

This reasearch suggests that native antizyme is post-translationally modified and this modification may play a role in the regulation of antizyme activity. Understanding antizyme form and function and its relation to the regulation of polyamine levels in both normal and diseased cells is critical to understanding breast cancer and tumor development.

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REGULATION OF CANCER CELL GROWTH BY C-TERMINAL HEXAPEPTIDE DERIVED FROM OSTEOCALCIN

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Osteocalcin (bone Gla protein) is a 49 amino acid protein synthesized by osteoblasts. It is an anchored component of the extracellular bone matrix. Osteocalcin limits bone formation based on studies of the Osteocalcin knockout mouse but the mechanism of action is not yet known.

Plasmin enzymatically produces an Osteocalcin-derived C-terminal hexapeptide (Hex, or Oc amino acids 44-49). Plasmin digestion of hydroxyapatite-bound Osteocalcin changes the properties of Osteocalcin. The two peptides produced, Hex and the remainder of the protein from the N-terminal to amino acid 43 (1-43) bind less effectively to hydroxyapatite. Plasmin cleavage would release soluble Hex from matrix-bound Osteocalcin. The 1-43 N-terminal peptide is present in the circulation, indicating that processing of Osteocalcin by plasmin may be a pathway to remove Ostecalcin during bone metabolism.

Hex promotes the growth of human osteosarcoma cells that are growth inhibited by the hormone oxytocin. Hex alone has no effect in serum-free media. However, it restores the growth of osteosarcoma cells which are growth inhibited by oxytocin. Hex appears to act only in the presence of oxytocin, implying it blocks the binding of the oxytocin to its receptor on osteosarcoma cells. The Hex sequence is homologous to the second extracellular domain of the oxytocin receptor which is thought to be essential for oxytocin binding.

Surprisingly, osteosarcoma cells express functional oxtocin receptors. Competitive binding studies have confirmed that Hex blocks the binding of oxytocin to receptor isolated from osteosarcoma cells.

Human breast cancer cells also express functional oxytocin receptors and are growth inhibited by oxytocin. It is plausible that Hex enhances the ability of breast carcinoma to metastasize to bone by providing a growth promoting effect similar to that afforded osteosarcoma cells. The knowledge could be useful for developing strategies to counteract the spread of breast cancer to bone.

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IDENTIFICATION OF PEPTIDE SEQUENCES THAT BIND HUMAN TELOMERASE HOLOENZYME

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The goal of this research was to identify peptide sequences that specifically and tightly bind human telomerase ribonucleoprotein (RNP). Random peptide bacteriophage display library technologies were used to identify such sequences. Specifically, 6- and 15-amino acid libraries, a generous gift from Dr. G. P. Smith (University of Missouri, Columbia, MO) were used in this study. Both libraries were constructed in a Fuse5 vector, where the foreign 6- or 15-amino acid peptide sequence is expressed on all 5 copies of the phage pIII minor coat protein.

Human telomerase RNP isolated from nuclear extracts of MDA-MB-435 breast carcinoma cells by a modified one-step affinity purification protocol was used as a target in the affinity selection procedures. Based on a protein concentration of the nuclear extract and eluted telomerase fractions, an estimated ≈ 15,000-fold degree of purification was achieved.

We performed three rounds of affinity selection using each library. The 136-fold and 147-fold enrichment, which were achieved between first and second rounds with 6- and 15-mer libraries correspondingly, were indicative of the successful isolation of the phages with affinity to human telomerase RNP. Because a high level of enrichment was achieved in the rounds 1 and 2, to increase selection stringency, in the third round, we decreased the amount of the target 5-fold compared with the amount used in the first two rounds.

To identify the 6- and 15-amino acid peptide sequences expressed on affinity-selected telomerase-avid phages, clone isolates from 6- and 15-mer libraries were individually propagated and subjected to DNA sequencing. The obtained DNA sequences were translated into 6- and 15-amino acid peptide sequences expressed on surfaces of the telomerase-binding phages.

In a course of this study, multiple peptide sequences exhibiting affinity toward human telomerase RNP were identified. Interestingly, some of the identified peptides express sequence similarities with proteins implicated in interaction with single-stranded telomeric DNA. The results of this research will provide a basis for the development of new line of peptide inhibitors of human telomerase, as well as for studies on its structural-functional characterization.

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UDP-GLUCURONOSYLTRANSFERASE-SPECIFIC GLUCURONIDATION INACTIVATES 4-HYDROXYTAMOXIFEN AND RALOXIFENE

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Interindividual variability of drug efficacy and toxicity may be attributed to common polymorphisms in genes encoding drug metabolizing enzymes. The selective estrogen receptor modulators (SERMs) 4-hydroxytamoxifen (OHT) and raloxifene (RAL) are metabolically inactivated by glucuronide conjugation. Glucuronidation reactions are catalyzed by the UDP-glucuronosyltransferases (UGTs), a family of endoplasmic reticulum-localized proteins that catalytically inactivate various endogenous and exogenous compounds. We hypothesized that UGT genetic polymorphisms contribute to the known variable clinical responses to OHT and RAL. The goals of this project were to identify the human UGT enzymes that catalyze glucuronidation of OHT and RAL and to identify genetic polymorphisms that might alter the activity of the gene products.

Radiochemical assays were used to determine that OHT is glucuronidated by UGT1A9 and UGT1A6, and RAL is glucuronidated by UGT1A9, UGT1A8, UGT1A1, and UGT1A7. DNA sequencing of UGT1A6 identified three non-synonymous single nucleotide polymorphisms defining four common alleles. UGT1A6 genotype correlated with UGT1A6 enzyme activity in a bank of human liver tissues. Through DNA sequencing of UGT1A9, polymorphisms near the putative promoter were identified, though no coding sequence polymorphisms were detected.

Our studies indicated that UGT1A9 is a critical enzyme for SERM metabolism and that UGT1A6 is also involved in OHT inactivation. We identified four UGT1A6 allelic variants that resulted in functionally distinct gene products. It is hoped that clinical applications of this knowledge will benefit prescribing of SERMs through genetic testing to optimize drug choice and dosage for patients.

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ENZYMATIC PROPERTIES OF MOUSE CYTOSINE DNA METHYLTRANSFERASE DNMT1

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DNA methylation in mammals is a part of gene regulation mechanism and aberrant DNA methylation is an early event in cancer development. Inhibition of DNA methylation in tumor cells has been shown to reverse tumor progression. We present the main catalytic features of Dnmt1, the principal methyltransferase in eukaryotic cells. Pre-steady state and steady state kinetics were used to analyze Dnmt1's catalytic preference for pre-methylated DNA, its processivity on DNA substrate, allosteric regulation, interaction with other proteins, and its likelihood to support mutagenic deamination. Dnmt1's preference for pre-methylated DNA substrate is due to faster target base attack, faster methyltransfer, and faster acid base catalysis in the active site. Dnmt1 can recognize DNA as pre-methylated even when methylated cytosine is one out of fifteen cytosines, however the enzyme is not self-activated early in methylation reaction. A novel analysis of processivity rate constants showed that Dnmt1 is more than 90% processive and its processivity can be regulated through its allosteric site. The extent of allosteric regulation depends on cofactor presence, DNA sequence and methylation status. DNA binding at the allosteric site inhibits Dnmt1 and initiates DNA release from its active site. S-Adenosylmethionine analogues and solvent kinetic isotope effect studies showed that enzyme mediated mutagenesis and cytosine deamination are due to enzyme's inability to protect its reactive intermediates. Presented results give molecular insights in DNA methylation and enable us to improve our current inhibitors of DNA methyltransferase.

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AN IMPROVED PERFUSION SYSTEM FOR NMR STUDY OF BREAST CANCER CELLS

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Ex vivo nuclear magnetic resonance is useful for studying the metabolic activity and the response of cells to chemotherapy agents. Such studies involve data acquisition over extended periods up to several days. It is important to preserve the integrity of cells during such prolonged studies by providing adequate nutrients and oxygen to the cells by perfusing aerated culture medium maintained at the normal physiological temperature of 37^{o}C. The presence of serum in the culture medium and temperature fluctuations can produce gas bubbles in the perfusion system. Air bubbles perturb magnetic field homogeneity in the NMR sample and causes line width broadening and loss of spectral resolution. We have utilized an improved perfusion system, which includes positive and negative pressure traps to prevent air bubble generation inside the NMR sample tube. This system has been used to prolong a typical 12 hour ex vivo study to longer than 10 days.

Wild type MCF-7 human breast cancer cells were grown to about 85% confluence in IMEM with 10% FBS. Approximately 1-2 x10^{8} cells were harvested and mixed with low temperature gelling agarose. The agarose-cell mixture was extruded into a NMR tube through a 0.5 mm i.d. Teflon tubing. This resulted in thin strands of spaghetti like threads containing cells embedded in agarose. These threads in the NMR tube were perfused with IMEM (0.9 ml/min) using a peristaltic pump, with strategically located air bubble traps before and after the pump and a reservoir for waste collection was located to collect the effluent from the NMR tube. A 400 MHz instrument was used with a repetition time 2 sec, flip angle 45^{0}, spectral width of 5000 Hz and 1800 transients were acquired over an hour. The ex vivo studies were performed at 37^{o} C and deuterium locked. Many phosphorus metabolites were identified on the basis of their chemical shifts and by spiking with the appropriate authentic compound, in particular phosphoethanolamine (PE), phosphocholine (PC), inorganic phosphate (Pi), glycerophospho-ethanolamine (GPE), glycerophospho-choline (GPC), phosphocreatine (PCr), γ-adenosine triphosphate (γ-ATP), α-adenosine triphosphate (α-ATP), diphosphodiester (DPDE), and β-adenosine triphosphate (β-ATP). The stability of the perfusion system was demonstrated over ten days by comparing a series of spectra obtained at one hour intervals.

In order to ascertain that the spectra represented the metabolic activity of viable cells, iodoacetamide (0.1 mM), a respiratory poison was perfused through the cells and the change in metabolite profile observed. The cytotoxic effect of iodoacetamide caused a decline in β–ATP peak over the next ten hours. Furthermore, signal for extracellular Pi appeared immediately upon perfusion with iodoacetamide. The peak for extracellular peak increased with concomitant decrease in the intracellular peak for Pi, suggesting leakage of intracellular Pi into the extracellular space. The improved cell perfusion is stable for several days and is reliable for the study of cell metabolism in viable cells and for monitoring the effects of cytotoxic agents.

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OXIDATIVE MECHANISMS IN ALCOHOL-INDUCED BREAST CANCER

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Alcohol consumption by women is one of several important risk factors for breast cancer (BC) that may promote BC through reactive oxygen species (ROS) induced carcinogenesis. Our preliminary studies had revealed that alcohol dehydrogenase (ADH), aldehyde oxidase (AOX) and xanthine oxidoreductase (XOR) are expressed and regulated in breast tissues. Mammary gland XOR and AOX were efficient sources of the ROS, hydrogen peroxide. Furthermore, XOR and AOX were found to generate ROS in two ways from alcohol metabolism: by a*cetaldehyde* consumption and by an *intrinsic NADH oxidase* activity of XOR and AOX. Importantly, genes for both XOR and AOX were found to be activated in mammary glands and mammary epithelial cells. The human XOR gene, encoding the ROS generating enzyme xanthine oxidoreductase, is activated in mammary epithelial cells by cytokines and hormones known to have significant affects in mammary gland development. Activation by cytokines was accompanied ROS generation that could be inhibited by XOR specific inhibitors. Thus, XOR has been found to be an inducible source of ROS in epithelial cells. The human AOX gene, encoding an enzyme highly related to XOR and capable of significant ROS generation was also found to be expressed in mammary epithelial cells. Furthermore, its gene was found to be activated by a group of transcription factors, the Sp family, known to be important for mammary gland development and to be of particular significance for alcohol metabolism. The potential for AOX and XOR to contribute to ROS generation in the mammary gland and ultimately to BC has been strengthened by the observation that both genes are activated in mammary glands and in mammary epithelial cells. Activation of each gene is distinct, therefore it is likely that they contribute to ROS generation at different times and following different stimulation. It is possible that that either AOX or XOR may contribute to ROS generation in the mammary gland during alcohol metabolism and possibly under additional circumstances.

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